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BRIEF COMMUNICATION

Latitudinal clines in bud flush phenology reflect genetic variation in chilling requirements in balsam poplar, *Populus balsamifera*

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PREMISE: Boreal and northern temperate forest trees possess finely tuned mechanisms of dormancy, which match bud phenology with local seasonality. After winter dormancy, the accumulation of chilling degree days (CDD) required for rest completion before the accumulation of growing degree days (GDD) during quiescence is an important step in the transition to spring bud flush. While bud flush timing is known to be genetically variable within species, few studies have investigated variation among genotypes from different climates in response to variable chilling duration.

METHODS: We performed a controlled environment study using dormant cuttings from 10 genotypes of *Populus balsamifera*, representing a broad latitudinal gradient (43–58°N). We exposed cuttings to varying amounts of chilling (0–10 weeks) and monitored subsequent GDD to bud flush at a constant forcing temperature.

RESULTS: Chilling duration strongly accelerated bud flush timing, with increasing CDD resulting in fewer GDD to flush. Genotypic variation for bud flush was significant and stratified by latitude, with southern genotypes requiring more GDD to flush than northern genotypes. The latitudinal cline was pronounced under minimal chilling, whereas genotypic variation in GDD to bud flush converged as CDD increased.

CONCLUSIONS: We demonstrate that increased chilling lessens GDD to bud flush in a genotype-specific manner. Our results emphasize that latitudinal clines in bud flush reflect a critical genotype-by-environment interaction, whereby differences in bud flush between southern vs. northern genotypes depend on chilling. Our results suggest selection has shaped chilling requirements and depth of rest as an adaptive strategy to avoid precocious flush in climates with midwinter warming.

KEY WORDS bud burst; endodormancy; climate; genotype-environment interaction; G×E; Salicaceae.

In northern climates, forest trees exhibit adaptive patterns of phenology in which they synchronize their growth and development with different environmental cues that mark the beginning and end of the growing season (Perry, 1971; Cooke et al., 2012). During mid-to-late summer, typically in response to decreasing photoperiod, trees cease height growth and set their buds in protective scales (Cooke et al., 2012). This is followed by cooler temperatures later in autumn that induce cold hardiness and dormancy (Howell and Weiser, 1970; Vitasse et al., 2014; Vitra et al., 2017), followed by the reinitiation of growth in the form of bud flush next spring

once a sufficient heat sum in the form of cumulative growing degree days (cGDD) has been met. Bud flush appears to be independent of photoperiod for *Populus* species, including *P. balsamifera* L. (Salicaceae) (Wareing, 1956; Soolanayakanahally et al., 2013); however, other northern temperate woody species are known to be photoperiod sensitive in the timing of spring phenology (Flynn and Wolkovich, 2018).

Because of its close link to climate change and warming temperatures, much research on the phenology of forest trees has focused on spring bud flush, which shows a well-documented trend of advancing bud flush timing (day of year) in response to earlier and warmer spring temperatures (Parmesan and Yohe, 2003; Peñuelas et al., 2009). In addition to these plastic responses, intraspecific variation in bud flush is known to be genetically heritable and under strong natural selection to take advantage of the available growing season while avoiding premature exposure to late-spring frosts, cold stress, and snow load (Savolainen et al., 2004; Way et al., 2011; McKown et al., 2014, Vitasse et al., 2014). Common garden studies in *Populus* also frequently show genetically based population differentiation and clinal variation in bud flush as a function of source latitude (Evans et al., 2016; Guerra et al., 2016; Keller et al., 2011; McKown et al., 2018). However, latitudinal clines measured in common gardens can range from strong to nonsignificant when compared across different planting sites or between different years within a site, even when measured on the same clonally propagated genotypes (e.g., Olson et al., 2013; Guy, 2014; McKown et al., 2018). This has been previously explained as environmental variation, emphasizing the role of genotype–environment interaction (G×E) in shaping intraspecific variation in bud flush, even when photoperiod remains the same from year to year and when the phenotype is standardized to heat sums (cGDD) instead of calendar day of year to account for interannual variation in the timing of spring temperatures (Guy, 2014).

Part of the complexity in unraveling the G×E response is that spring bud flush is actually the culmination of two distinct, yet interacting, underlying physiological processes—rest and quiescence—which operate in conjunction to control plant responsiveness to temperature at different stages of dormancy (Samish, 1954; Lundell et al., 2019). Plants first enter a state of rest once buds are fully hardened at the end of the previous growing season and will remain in a state of rest unless they experience an accumulation of near-freezing temperatures, often quantified as cumulative chilling degree days (cCDD), which promotes the process of rest break. Once sufficient chilling has accumulated and the bud reaches a state of rest completion, typically by midwinter, plants transition to a state of quiescence, where ontogenetic growth within the bud occurs at a rate dependent on the air temperature (some species also possess an interaction with photoperiod) until the growth causes bud flush (Heide, 1993; Lundell et al., 2019). However, the transition between rest and quiescence can be gradual and nondiscrete, with chilling needs still being met at the same time that heat sums start to accumulate, resulting in an overlap of these two stages (Way, 2011; Guy, 2014; Lundell et al., 2019).

For many species of forest trees, information on chilling requirements and rest completion dates are lacking (Chuine et al., 2016) and even among closely related species, chilling needs can differ greatly (Man et al., 2017). For example, in a recent multispecies study, Man et al. (2017) found that *Populus tremula* propagated via root suckers require ~400 chilling hours (~17 cCDD), while *Populus balsamifera* propagated through stem cuttings require ~200 chilling hours for normal bud flush. Interspecific comparisons have shown that species vary in how chilling duration affects the degree of rest break and the timing of bud flush, with inadequate chilling resulting in delayed bud flush (Polgar and Primack, 2011; Man et al., 2017; Nanninga et al., 2017). If the same holds true within species, this could explain the year-to-year variability in latitudinal clines observed in common garden studies, in which timing of bud flush may depend on genotypic differences among source climates in how the extent of rest break affects the rate of ontogenetic growth in quiescent buds (e.g., McKown et al. 2018; Delpierre et al.,2019). Several studies have investigated how genotypic variation for chilling requirements affects bud flush (Campbell and Sugano, 1975; Cannell and Willett, 1975; Cannell and Smith, 1983; Hannerz et al., 2003; Harrington et al., 2010; Dantec et al., 2014; McKown et al., 2018), but to our knowledge, only Myking and Heide (1995) and McKown et al. (2018) have specifically addressed the question of context-dependency of latitudinal clines on the amount of chilling (with photoperiod sensitive and insensitive species, respectively). Myking and Heide (1995) showed a decrease in time to bud flush with increasing source latitude. McKown et al. (2018) identified a quadratic relationship, in which the most southerly and northerly genotypes showed the earliest bud flush, while genotypes from mid-latitudes showed later bud flush.

Here, we report on a controlled environment study in which we exposed dormant cuttings from balsam poplar (*Populus balsamifera*) genotypes originating from a latitudinal gradient to a continuous range of chilling duration to test for the influence of G×E interaction on bud flush. Previous work in *P. balsamifera* has shown that bud flush is heritable (Keller et al., 2011; Olson et al., 2013) and shows latitudinal clines that vary among common garden sites (Olson et al., 2013; Guy, 2014), making it a strong candidate for studying variation in bud flush. We extend this work by testing the hypothesis that clines in bud flush arise because of genotypes from different source climates varying in how degree of rest break, as a result of cCDD, interacts with forcing temperatures to determine the timing of bud flush. Specifically, we predict that southern genotypes require greater cCDD to reach rest completion compared to northern genotypes, resulting in clines in bud flush when cCDD is minimal, as seen in other studies (e.g., McKown et al., 2018).

MATERIALS AND METHODS

Study species

Balsam poplar (*Populus balsamifera*) is a widely distributed boreal deciduous tree, with a geographic range that spans over 30° of latitude, from Colorado, USA to the Yukon Territory, Canada, and longitudinally from Alaska, USA to Newfoundland, Canada (Little, 1971). Along with its sister species, *P. trichocarpa* (Tuskan et al., 2006), poplars have emerged as a model system for studying the genetic and phenotypic basis of locally adaptive phenology across broad climatic gradients (Keller et al., 2011; Olson et al., 2013; Soolanayakanahally et al., 2013; Evans et al. 2014; McKown et al. 2018; Zhang et al., 2019).

Plant tissue

We employed the established technique of using dormant vegetative cuttings with terminal and/or lateral buds to measure the response

of bud flush timing to degree of rest break achieved by varying amounts of cCDD (Primack et al., 2015). The cuttings were collected from dormant trees that had grown for three years in a raised bed common garden located outside of Jeffords Hall at the University of Vermont (Burlington, Vermont, USA, 44.4759°N, 73.2121°W, 61 m a.s.l.). This common garden was planted in October 2014 from cuttings obtained from the larger Agriculture Canada Balsam Poplar (AgCanBaP) collection of trees sampled from natural populations (for additional details on the original source collections, see Table 1, Soolanayakanahally et al., 2009, 2013). By growing cuttings for three years in a common garden prior to initiating our experiment, we sought to minimize the influence of any nongenetic environmental effects attributable to the original source climates. For this study, we sampled cuttings from 10 genotypes (two each from five populations; Table 1) spanning 15° of latitude (43–58°N), 65° of longitude (57–122°W), and all three regional genetic clusters identified by Keller et al. (2010).

Measurement of bud flush under experimental chilling and heating

Our experimental design consisted of exposing cuttings to a variable number of weeks spent chilling at a constant temperature of 4°C, followed by transfer to a growth chamber set to a constant forcing temperature of 10°C and a 24 h photoperiod. In doing so, we varied the cumulative chilling received by the cuttings and by extension the degree of rest break achieved and then quantified the cumulative heat sums until bud flush was observed. By using constant chilling and forcing temperatures, the absolute time spent chilling or in the growth chamber leading up to flushing by the cuttings was linearly proportional to the accumulated CDD and GDD, respectively. While bud flush phenology in *Populus* is thought to be primarily under the control of temperature (Soolanayakanahally et al., 2013), our use of a constant 24 h photoperiod standardized any potential interactions of photoperiod with bud flush timing (see also McKown et al., 2018).

Starting on January 4, 2018, cuttings were taken from the common garden, immediately sealed in plastic bags, and kept in dark storage in a cold room at 4°C. On January 5, 2018 and continuing every week for 10 weeks (11 total weeks), four cuttings from each genotype with healthy buds were taken out of the cold room, for a total of 40 cuttings per week, or 440 cuttings experiment-wide. Cuttings were surface sterilized by soaking in a 0.1% solution of Green-Shield II (BASF, Ludwigshafen, Germany) (10% ammonium chloride and 10% dimethyl ethylbenzyl ammonium chloride) for 10 minutes. Cuttings were given fresh cuts at least 2 cm from the base and planted in 164 mL Cone-Tainer pots (Steuwe and Sons, Tangent, Oregon, USA) filled with thoroughly wetted ProMix BX potting soil (Premier Tech, Quebec, Canada). Cuttings were ~15 cm in length and planted to a depth that ensured that at least two buds (either one terminal and one lateral bud, or two lateral buds) were above the surface of the potting soil. Planted cuttings were placed into a PGR15 growth chamber (Conviron, Winnipeg, Canada) at 10°C, 24 h light, and 420 $\mu mol \cdot m^{-2} \cdot s^{-2}$ to accumulate heat sums and stimulate bud flush. It is important to note that just after the Week 6 cohort of cuttings was planted, a growth chamber malfunction resulted in temporary darkness and an increase in temperature (to ~23°C) for 4 h before returning to programmed settings.

Bud flush was checked daily at the same time and scored according to stage three from Soolanayakanahally et al. (2013), indicated by a clear separation of bud scales and visible protrusion of leaves from the buds. We recorded the Julian day that each cutting flushed, which was then converted to cumulative growing degree days (cGDD) using the equation $(T_{max} + T_{min})/2 - T_b$ assuming a base temperature (T_b) of 0°C (Olson et al., 2013). Similarly, we converted chilling weeks to cumulative chilling degree days (cCDD) based on Equation 1 of Kramer (1994), where chilling units accumulate at temperatures greater than –3.4°C (T_{min}) and less than 10.4°C (T_{max}), with an optimal accumulation at 3.5°C (T_{opt}).

$$CDD * day^{-1} = \begin{cases} 0 & T \le T_{min} \\ \dfrac{T - T_{min}}{T_{opt} - T_{min}} & T_{min} < T \le T_{opt} \\ \dfrac{T - T_{max}}{T_{opt} - T_{max}} & T_{opt} < T < T_{max} \\ 0 & T \ge T_{max} \end{cases}$$

To account for chilling that occurred outside prior to our sampling on January 4, 2018, we downloaded daily temperature data from Burlington International Airport (South Burlington, Vermont, USA, 44.4707°N, 73.1516°W, 102 m a.s.l. and applied Equation 1 of Kramer (1994) to calculate cCDD from September 1, 2017 (after all genotypes had set bud, but prior to accumulation of chilling temperatures) until January 4, 2018. By the start of the experiment, all genotypes had experienced accumulated ambient chilling of 23.07 cCDD before being transferred to the cold

TABLE 1. Location and climate of origin for 10 genotypes of *P. balsamifera*.

Location[1]	Genotype	Latitude	Longitude	Elevation (m)	MAT (°C)[2]	Mean cCDD[3]	Mean WGDD[4]
London, ON	LON_03	43.14	–81.75	338	7.8	68.1 ± 12.2	108.8 ± 67.0
London, ON	LON_11	43.04	–81.18	338	7.3	65.2 ± 12.3	100.5 ± 64.3
Chapleau, ON	CPL_03	47.50	–83.25	444	1.7	54.5 ± 11.3	21.2 ± 30.9
Chapleau, ON	CPL_10	47.51	–83.26	444	1.7	55.1 ± 11.4	21.1 ± 30.9
Hawkes Bay, NL	HWK_11	50.37	–57.10	11	1.0	58.4 ± 12.8	3.9 ± 5.0
Hawkes Bay, NL	HWK_14	50.53	–56.57	11	1.2	68.0 ± 13.1	3.6 ± 4.8
Saskatoon, SK	SKN_05	52.34	–106.16	518	1.7	49.4 ± 11.8	14.9 ± 16.6
Saskatoon, SK	SKN_10	52.45	–107.04	518	1.7	49.8 ± 11.2	19.0 ± 19.3
Fort Nelson, BC	FNO_12	58.50	–122.51	414	–1.5	45.4 ± 10.6	10.0 ± 11.3
Fort Nelson, BC	FNO_15	58.49	–122.50	414	–1.4	45.5 ± 10.5	10.0 ± 11.3

Notes: [1]All locations are in Canada and territory abbreviations are as follows: ON, Ontario; NL, Newfoundland; SK, Saskatchewan; BC, British Columbia.
[2]Mean annual temperature derived from WorldClim (1970–2000).
[3]Mean cumulative chilling degree days (cCDD) until the start of spring estimated from MODIS satellite remote sensing. Means ± 1 SD are from daily meteorological data from 2001–2016.
[4]Mean winter growing degree days (WGDD), estimated as cGDD with T_b>0 during the period of Jan01–Mar30. Means ± 1 SD are from daily meteorological data from 2001–2016.

room, after which they experienced a constant rate of chilling of 0.93 cCDD per day for the duration of their treatment. Once they were moved to the growth chamber to force bud flush, they accumulated 10 GDD per day.

Historical cCDD were calculated and averaged over 16 years for each genotype's source location based on daily meteorological data available from the Daymet climate database (https://daymet.ornl.gov) and using the estimated start of spring phenology using satellite remote sensing data (details described in Elmore et al., 2016). Briefly, based on the Moderate Resolution Imaging Spectrometer (MODIS) Vegetation Dynamics product for 2000–2016, the latitude and longitude of each genotype was used to assign a yearly start of spring (SOS) date in Julian days, which corresponds to the inflection point of the rapid green-up of that pixel in remotely sensed vegetation after the longest period of no/low remotely sensed vegetation, which can be used as a proxy for bud flush (Ahl et al., 2006). For each genotype-year combination, the daily average temperatures from Julian day 1 to the SOS date and from Julian day 244 (September 1) to 365 of the previous year, were used to calculate cCDD based on Equation 1 of Kramer (1994). The cCDD were averaged and standard deviation calculated across the 16 years for each location to get the typical cCDD experienced in the source location of each genotype. Similarly, we used Daymet climate data to estimate historical values for the accumulation of winter growing degree days (WGDD) for each genotype. We used the same formula for calculating WGDD as in cGDD (with $T_b = 0$), but limited the seasonal period to midwinter (Jan 01–Mar 30). Thus, WGDD quantifies historic exposure of each genotype's source site to forcing temperatures capable of triggering bud flush.

Data analysis

We analyzed variation in bud flush with linear mixed-effects models using the *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017) packages in R (R Core Team, 2018). We tested the response of cGDD to bud flush as a function of the fixed effect of chilling duration (cCDD). Bud flush variation among genotypes was modeled as either a random intercepts model (1|genotype) that tested for overall genotypic variation (G), or as random intercepts and slopes model that tested for a G×E interaction with chilling (1 + cCDD|genotype). In a second group of models, we included source latitude and its interaction with chilling as fixed effects. We performed model selection between different nested models using likelihood ratio tests. Initial tests showed no nonlinearity in bud flush response to chilling based on fitting quadratic models to the data to test for thresholds where more chilling does not result in a further decrease of GDD to bud flush (result not shown). Because of limited replication, we did not test for differences among the regional genetic clusters identified in Keller et al. (2010), but also note that previous studies showed no significant differences in bud flush timing among these regions (Keller et al., 2011).

Lastly, we used the R package *MCMCglmm* (Hadfield, 2010) to calculate Bayesian estimates of broad-sense heritability ($H^2 = V_G/V_P$), where V_G is the variance among clonally propagated genotypes and V_P is the total phenotypic variance (= V_G + residual error variance). To investigate if the magnitude of genetic and phenotypic variance components changed with chilling duration, we calculated H^2, V_G, and V_P separately for each week of chilling. Bayesian models used uniform priors and were run for 2×10^5 iterations after a burn-in of 5×10^4 and thinning interval of 50 iterations.

RESULTS

Genotypes display a clear trend towards decreasing cGDD to bud flush with increased cCDD with genotypic variation relatively constant in rank order across chilling treatments (Fig. 1A). Chilling had a strong, negative effect on the timing of bud flush after controlling for genotypic variance ($\beta = -1.97$; $P < 0.0001$), indicating a decrease in roughly two cGDD needed to flush for each additional cCDD (Table 2, Model 1). Including the random effect of G×E interaction with cCDD (Model 2) provided a significantly better fit compared to just the random effect of genotype, increasing the variance in bud flush explained from 64% to 67% (Table 2). Several genotypes (CPL_03, CPL_10, LON_03, and LON_11) showed a notable deviation from the overall trend at Week 6 of chilling (62.13 cCDD) when the growth chamber malfunctioned,

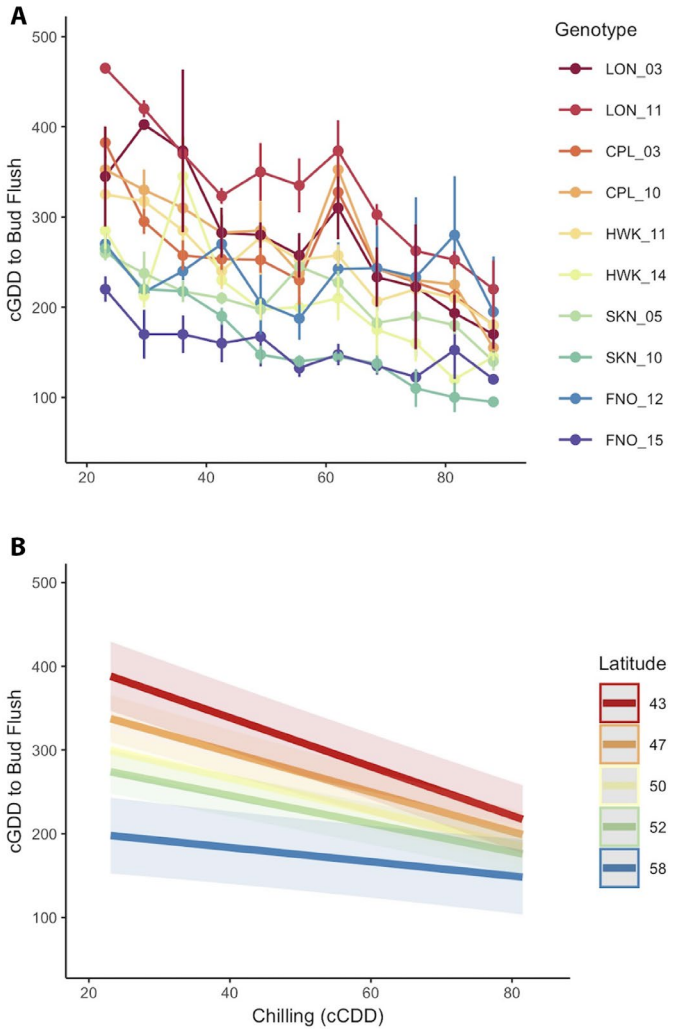

FIGURE 1. Genetic variation for cumulative growing degree days, cGDD, to bud flush as a function of cumulative chilling degree days, cCDD. One day at forcing temperatures in the growth chamber is equivalent to 10 GDD. The cuttings were harvested with 23.07 cCDD and once in the 4°C room accumulated 0.93 CDD per day. (A) Genotypic means ± SEM; (B) mixed-effects model prediction showing interaction between genotype source latitude and chilling duration. Colors in both plots reflect the gradient in source latitude.

TABLE 2. Linear mixed-effects models of cGDD to bud flush.

		Fixed Effects (coefficient ± SEM)			Random Effects (SD)		
	R^2	cCDD	Latitude	cCDD * Lat	G[1]	G×E[2]	Residual
Model 1	0.64	−1.97 ± 0.13***	—	—	55.03	—	51.07
Model 2	0.67	−1.95 ± 0.27***	—	—	85.91	0.75	49.05
Model 3	0.66	−8.88 ± 1.26***	−15.88 ± 2.69***	0.14 ± 0.03***	35.91	—	49.17
Model 4	0.82	−1.95 ± 0.27***	−16.09 ± 1.78***	—	16.24	0.77	49.05

Model Selection Likelihood Ratio Tests:
Model 1 v. Model 2: LRT $\chi^2_{df=2} = 18.2$; $P < 0.001$
Model 1 v. Model 3: LRT $\chi^2_{df=2} = 39.16$; $P < 0.001$
Model 2 v. Model 4: LRT $\chi^2_{df=1} = 11.67$; $P < 0.001$

Notes: *** $P < 0.001$.
[1]G effects: random intercepts among genotypes (1|genotype).
[2]G×E effects: random intercepts and slopes with cCDD among genotypes (1 + cCDD|genotype).

but subsequent cohorts followed the mean trend. Interestingly, those genotypes most responsive to this event originated from the most southerly latitudes in our sample (Table 1), but the results excluding the Week 6 cohort were highly similar to the full data set (Table 2; Appendix S1).

Including the source latitude of genotypes and the interaction of latitude with cCDD significantly improved the model fit compared to the equivalent model without latitude (Table 2, Models 1 vs. 3). Latitude affected cGDD to bud flush both singly ($\beta = -15.88$; $P < 0.0001$) and through an interaction with cCDD ($\beta = 0.14$; $P < 0.0001$). The direction of the latitude effect revealed a cline, in which more northerly genotypes required fewer cGDD to flush compared to more southerly genotypes (Fig. 1B), consistent with differences in the average accumulation of chilling degree days among source sites (Table 1; Appendix S2). Notably, the interaction of latitude with cCDD caused the cline in bud flush to be most evident when chilling was minimal, and as chilling accumulated the timing of bud flush converged among latitudes (Fig. 1B). Adding an additional random effect of G×E to this model prevented convergence, probably because the G×E interaction with chilling was strongly correlated with the interaction between each genotype's source latitude and chilling. Accordingly, by dropping the cCDD*latitude interaction, we were able to fit a G×E random effect (Model 4) that was highly significant and provided a better fit over the equivalent Model 3 that lacked latitude as a predictor ($\chi^2 = 11.67$, $P < 0.0001$). Similar results were obtained when latitude was replaced with the predictors mean annual temperature, mean temperature of the coldest quarter, and WGDD (Appendices S1 and S3). However, these temperature predictors provided a slightly poorer fit to the data compared to latitude in explaining the nature of G×E variation in bud flush.

Broad-sense heritability of bud flush was high at the start of the experiment (Week 0), with a median posterior H^2 estimate of 0.714 (95% highest posterior density interval = 0.423 − 0.937). Heritability remained at high levels throughout most of the chilling treatments and was not correlated with chilling duration ($r = -0.423$, $P = 0.194$), although there was some indication of a decrease in the final three weeks of chilling (Fig. 2). In contrast, negative correlations with chilling duration were observed for both V_G ($r = -0.631$, $P = 0.037$) and V_P ($r = -0.619$, $P = 0.042$) (Fig. 2). Thus, the magnitude of both genetic and phenotypic variance in bud flush significantly decreased with increased chilling (Fig. 1A), although the ratio of V_G/V_P remained fairly constant.

DISCUSSION

By experimentally controlling the accumulation of chilling and using genotypes sampled from across a latitudinal gradient and grown under controlled environmental conditions, our study explicitly teased apart effects of chilling, genotype, and G×E for bud flush timing. The results confirmed a primary role for chilling duration lessening the cGDD needed to flush, consistent with prior studies on *Populus* and other woody species (e.g., Cooke et al., 2012; Nanninga et al., 2017, McKown et al., 2018). However, the significant G×E effect also confirms that genotypes varied in the degree of rest break induced by the chilling treatments, with the accelerating effect of chilling on bud flush dependent upon genotypic background. While all genotypes displayed quiescence and flushed under increasing cGDD, genotypes from southern latitudes required more cCDD to reach a degree of rest break that yielded bud flush timing comparable to genotypes from northern latitudes.

Our findings suggest that latitudinal clines in bud flush phenology reflect genotypic variation in depth of rest as an adaptive strategy to avoid precocious bud flush in climates that experience a higher likelihood of midwinter warming. This hypothesis is also consistent with environmental variation in the amount of chilling accumulation in an average year, which acts as the mechanism to break rest (Table 1; Appendix S2). Pervasive cold winter temperatures experienced by northern genotypes maintain no or low ontogenetic growth rates during quiescence and remove the need for a deep rest broken by chilling accumulation, allowing rapid onset of ontogenetic growth upon the return of warm temperatures in spring. In contrast, southern genotypes likely evolved deeper rest to stave off bud flush during winter warm spells, requiring greater chilling accumulation to break rest thus preventing premature bud flush during midwinter warm periods (Howe et al., 2003).

Other temperate-boreal tree species have been shown to display genetically-based latitudinal clines in phenology traits (e.g., seasonal phenology, winter dormancy and chilling requirements; Heide, 1993), consistent with selection acting on depth of rest. For example, Myking and Heide (1995) found northern provenances of two species of Scandinavian birch (*Betula pendula* and *B. pubescens*) had decreased chilling requirements relative to southern provenances, and a similar result was reported for Norway spruce (*Picea abies;* Hannerz et al., 2003). In poplars, McKown et al. (2018) recently reported the presence of positive latitudinal clines in bud flush in common garden and growth chamber

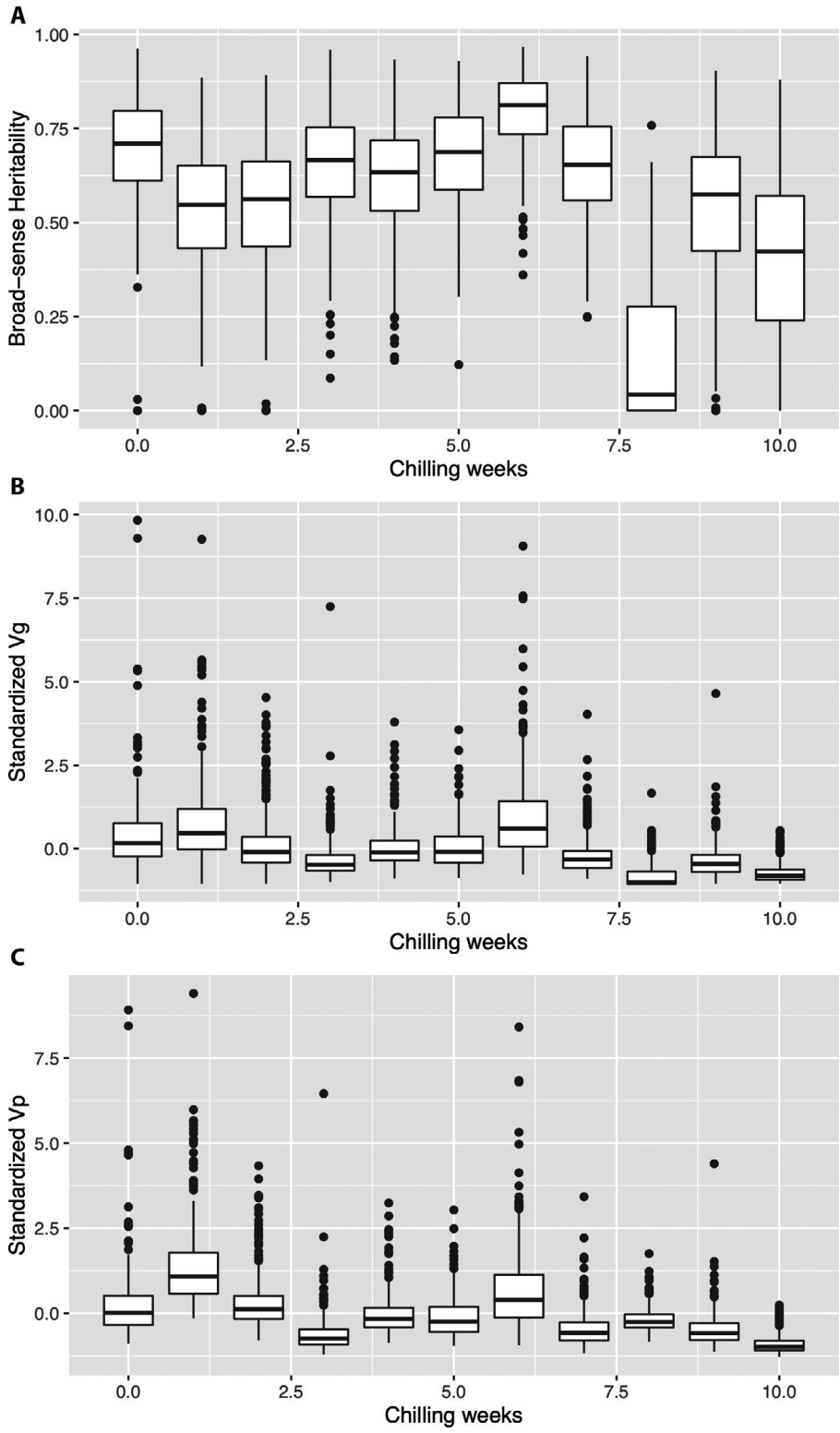

FIGURE 2. Bayesian posterior estimates of quantitative genetic variance in cumulative growing degree days (cGDD) to bud flush estimated per week of accumulated chilling degree days (cCDD): (A) broad sense heritability, (B) genotypic variance, (C) phenotypic variance. Genotypic and phenotypic variance decreased with increased cCDD (linear regression: V_G, $r = -0.631$, $P = 0.037$; V_P, $r = -0.619$, $P = 0.042$).

studies of *P trichocarpa*—the sister species to *P. balsamifera*. The cGDD to bud flush in *P. trichocarpa* increased linearly with the source latitude of genotypes, although when the most northerly genotypes in their sample (>56°N) were included, there was evidence for a quadratic relationship across the range showing a decrease in bud flush timing of these northern genotypes (McKown et al., 2018). However, when experimental chilling was extended to 98 days, the cline was greatly reduced because most genotypes flushed quickly upon transfer to forcing temperatures, similar to what we observed under our longest treatment of chilling.

While McKown et al. (2018) and our study focused on closely related species sampled across a similar latitudinal gradient (~43–58°N), there are numerous differences between the two studies. Most notably, McKown et al. (2018) sampled many more genotypes tested under two discrete chilling treatments, whereas we sampled relatively few genotypes exposed to a more continuous range of chilling duration. Given these differences in experimental design, both studies were remarkably congruent in showing that genetic variation in bud flush timing is highly sensitive to the extent of chilling, forming a G×E×E interaction whereby increased chilling lessens the heat sums necessary to flush in a manner that varies among genotypes from different latitudes. While both sister species show strong quantitative genetic clines in phenology along climatic gradients (e.g., Keller et al., 2011, Olson et al., 2013, Soolanayakanahally et al., 2013, Evans et al., 2014, McKown et al., 2014), they also occupy distinct climate niches (Levsen et al., 2012) and likely have experienced different selection pressures on vegetative phenology. The reduced chilling needs of the most southerly genotypes in McKown et al. (2018) could be explained by local selection under the warm maritime-influenced climate of southern *P. trichocarpa* favoring genotypes that require minimal chilling, whereas at equivalent latitudes the climate of southern *P. balsamifera* genotypes is cooler and more continental, selecting for greater chilling requirements.

Local adaptation of chilling levels required for rest break requires genetic

variation for selection to act on. While multiple studies have reported significant broad-sense heritability (H^2) for bud flush in common garden studies of *Populus* (Keller et al. 2011; McKown et al., 2014; Evans et al., 2016; McKown et al., 2018), these estimates generally confound variation attributable to chilling vs. the rate of ontogenetic growth. In our study, broad-sense H^2 was high and relatively constant across chilling treatments, although the lowest values were observed under the longest chilling treatments (Figure 2). Both genotypic and phenotypic variance were also greatest under minimal chilling where degree of rest break for most genotypes was incomplete, and declined with increased chilling duration. This suggests that genetic variation in bud flush may be attributable to genotypic differences in depth of rest, and once rest has been satisfied, phenotypic variance increasingly reflects environmental causes. Our broad-sense H^2 estimates agree well with previous reports (H^2 = 0.47–0.81; Olson et al., 2013) but are higher than the narrow-sense heritability (h^2) reported by Keller et al. (2011) (h^2 = 0.25, 0.15–0.70, 95% CI). This may be expected because H^2 contains additional nonadditive components of genetic variance (dominance, epistasis, G×E), unlike h^2. Regardless, all three studies agree on the importance of spatial structure (e.g., population or latitudinal differences) in explaining the genetic variance in bud flush. These results, together with the convergence in cGDD to bud flush among genotypes from different latitudes (Fig. 1B), is consistent with the hypothesis that latitudinal clines reflect local selection on chilling requirements under contrasting climates.

The implications of our findings have relevance for understanding and predicting how phenology will be affected by degree of rest break, and the role of warming winter and spring temperatures in species that rely primarily on temperature cues for bud flush. With rising winter temperatures due to climate warming, the probability that chilling requirements are not adequately being met has increased, especially at southerly latitudes (Table 1; Guy, 2014), which may result in erratic bud flush patterns with poor new growth and delayed bud flush timing even under a warmer overall climate (Yu et al., 2010; Hänninen and Tanino, 2011). Expanding our knowledge on the role of genetic variation and environmental drivers of phenology like CDD and GDD, as we have done here, is therefore important in refining predictive models to understand how temperate-boreal forests will react to climate change.

When our growth chamber malfunctioned, we observed a delay in bud flush timing. Although bud flush in *Populus* species is generally considered to be photoperiod insensitive (Wareing, 1956; Soolanayakanahally et al., 2013; McKown et al., 2018), the effect of elevated temperature would be expected to *accelerate* the time to bud flush by increasing the GDD accumulated, so the delay in bud flush may suggest an unexpected response to the sudden darkness breaking the otherwise 24 h photoperiod. There is evidence in other woody species to show that in cases of insufficient chilling, long-day photoperiod can play an important role in bud flush (Worrall, 1983; Polgar and Primack, 2011; Flynn and Wolkovich, 2018). Whether photoperiod can partially compensate for insufficient chilling in *Populus,* and whether this varies latitudinally within species, points to an interesting avenue for future investigation (Chandler and Thiegles, 1973; Guy, 2014).

CONCLUSIONS

We have demonstrated that intraspecific genetic variation in chilling requirements within *P. balsamifera* contributes to a latitudinal cline in bud flush, likely as a result of local selection in response to geographically varying winters in the Northern Hemisphere acting on the depth of bud rest. Species-specific studies that elucidate genetic variation of how degree of rest break by cCDD interacts with warm forcing temperatures to dictate timing of bud flush among genotypes from different climatic source environments are needed for building more realistic phenology models. Our study adds to the existing literature emphasizing the importance of understanding the underlying processes of exiting dormancy that are crucial for the survival of temperate and boreal woody species. Future studies should search for candidate genes underlying genetic variation for chilling requirements, ultimately leading to a better understanding of how species respond to dramatically different future climates.

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AUTHOR CONTRIBUTIONS

E.T. and S.K. planned, designed, and executed the experiment, and analyzed the data. E.T., S.K., and R.S. wrote the manuscript.

DATA AVAILABILITY

Data and scripts are available on S.K.'s GitHub account: https://github.com/stephenrkeller/Pbalsamifera_chilling.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Linear mixed-effects models of cGDD to bud flush using different source climate predictor variables.

APPENDIX S2. Historical midwinter climate trends for each genotype from 2001–2016.

APPENDIX S3. Linear mixed effects models of cGDD to bud flush as a function of the interaction between cCDD and mean annual temperature, mean temperature of coldest quarter, and WGDD.

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